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DEVELOPMENT OF PLAQUE ASSAY SYSTEMS FOR POLIOVIRUS.(U)
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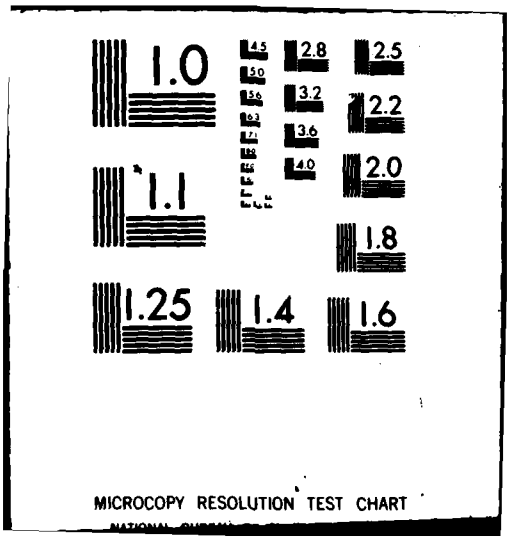
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# SUFFIELD MEMORANDUM

NO. 1067

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by

R.E. Fulton and K. Munsoe





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**Abstract**

During the summer months of 1978, Ms. Krista Munroe was employed as a summer research assistant in the Department of Preventive Medicine, DRES. Her project was to develop a plaque assay system for the quantitation of infectious poliovirus type 1. Two different plaque assay techniques were developed and compared. The results of this work are presented in this Suffield Memorandum.



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## Introduction

The Defence Research Establishment Suffield (DRES) large volume air sampler (LVAS) (1) is efficient in the collection of bacteria from air (2). This equipment is presently being considered as a means of collecting viruses from air. To properly evaluate the efficiency of the LVAS for virus collection, the development of a simple, reliable and efficient virus assay technique is required. To date, the plaque assay offers the most economical and reliable method for quantitation of infectious virus.

The usefulness of a plaque assay for quantitation of airborne viruses is dependent upon the suitability of a particular host biological system for isolation of a specific virus. Since host cells vary in their susceptibility to viruses, optimal conditions for host-cell-virus interaction must be ascertained for each type of virus to be collected and assayed.

The vaccine strain of poliovirus type 1 (Sabin) was chosen as a suitable test virus for initial evaluation of the efficiency and sensitivity of the LVAS for collection of viruses. This virus was chosen because it grows well in cell culture, its quantitation by plaque assay has been previously documented (3,4,5), and it is a relatively safe virus with which to work.

This report describes and compares two plaque assay techniques:

1. a modification of the technique used by Dr. C.M. Johnson-Lussenburg, University of Ottawa (personal communication), and

2. a modification of techniques as described by Stott et al. (6) and Killington et al. (7).

Results of an evaluation of commercially available cell culture Petri dishes for their suitability for use in plaque assays is also presented.

## MATERIALS AND METHODS

### Cell Cultures

A continuous line of human embryonic lung carcinoma cells (L 132), kindly supplied by the University of Ottawa, were grown as monolayers in Earle's Minimum Essential Medium (MEM) (Flow Laboratories, Mississauga, Ontario), containing 10% fetal calf serum (FCS) (v/v), penicillin (P)/gentamicin (G) (10,000 I.U. per mL/0.2 mg per mL), and glutamine (0.29 g per L). Cells were cultured in 250 mL plastic tissue culture flasks (Falcon, Fisher Scientific, Edmonton, Alberta). When monolayers were established growth medium was removed and the cells washed twice with phosphate buffered saline (PBS) (incomplete Dulbecco; Med-Ox Chemicals, Ottawa, Ontario). Cells were then trypsinized with trypsin-EDTA (GIBCO, Burlington, Ontario) and resuspended in MEM growth medium. Plastic Petri dishes (60 x 15 mm) were seeded with five mL of the cell suspension diluted to contain $1.75 \times 10^5$ cells/mL. The cells were incubated overnight or until confluency in an atmosphere of 5% $CO_2$ and 55% relative humidity. Cell culture Petri dishes from three commercial sources were evaluated for their suitability for plaque assays. Linbro® multi-dish four well Disposo plates were obtained from ICN, Montreal, Quebec. Falcon® single well culture dishes were obtained from Fisher Scientific, Edmonton, Alberta. LUX Contur® single well dishes were purchased from Microbiological Associates, Walkersville, Maryland.

### Virus

Virus seed stock was supplied by the University of Ottawa. This virus was initially described as poliovirus type 1 (Sabin) but was later

determined by virus neutralization to be a mixture of poliovirus types 1 and 3. This virus seed was passaged six times in L 132 cells, then harvested and held at -90°C until required. The passaged virus was titrated by dilution end-point and the titer calculated using the Kärber method (8). The titer, expressed as 50% tissue culture infective dose ($TCID_{50}$), was $10^{-7}$/0.1 mL.

## Plaque Assays

### Method I

This method was modified from a procedure obtained from Dr. C.M. Johnson-Lussenburg, University of Ottawa (personal communication).

Growth medium was removed from culture dishes containing confluent monolayers of L 132 cells and the cell sheets were washed twice with PBS containing 0.2% (v/v) bovine serum albumin (BSA) (PBS-BSA). Virus dilutions were prepared on ice in PBS-BSA and cultures were inoculated in duplicate with 0.1 mL of each appropriate virus dilution. The virus was allowed to adsorb for one hour on a level surface at 37°C in a humidified atmosphere (55%) containing 5% $CO_2$. The inoculum was respread every 30 minutes by gentle tipping of the culture dishes. Excess inoculum was removed and five mL of agar overlay was added to each dish. The agar overlay medium consisted of the following final concentration of ingredients: 2X MEM; 0.2% $NaHCO_3$; 200 µg/mL diethylaminoethyl-dextran (DEAE-dextran) (Pharmacia, Dorval, Quebec); 50 µg/mL bromodeoxyuridine (BUDR); 100 I.U./mL P, 0.2 µg/mL G; 30 mM $MgCl_2$; 0.29 g/L glutamine; 2.0% FCS; 0.6% Ionagar #2 (Oxoid, Med-Ox Chemicals, Ottawa, Ontario); and sterile distilled water. The agar overlay was allowed to solidify on a

level surface at room temperature, after which the cultures were incubated for 72 hours at 37°C in a humid environment flushed with 5% $CO_2$. The following controls were included:

1. cultures mock infected and overlayered with agar medium,
2. cultures mock infected and overlayered with liquid medium containing all components of agar medium except agar,
3. cultures inoculated with lowest dilution of virus and overlayered with liquid medium containing all components of agar overlay except agar, and
4. cultures inoculated with lowest dilution of virus and overlayered with liquid medium consisting of MEM containing 2% FCS.

At the end of the incubation period, cultures were fixed by adding 5 mL of 4% formaldehyde in saline to the agar surface and incubating for one hour at room temperature. The agar layer was then removed and the cell sheets stained for 15 minutes with 1% aqueous crystal violet. Excess dye was washed off with tap water and the stained cell sheets allowed to air dry. Plaques were counted on the same day and the titer calculated.

Method II

This method was modified from procedures described by Stott et al. (6) and Killington et al. (7).

Growth medium was removed from confluent monolayers of L 132 cells and the cell sheets washed twice with PBS-BSA. Virus dilutions were prepared on ice in PBS-BSA and cultures were inoculated in duplicate with 0.1 mL of appropriate virus dilution. The virus inoculum was allowed to

adsorb on a level surface for 2.0 hours at 37°C in an atmosphere of 5% $CO_2$ and 55% relative humidity. The inoculum was respread every 30 minutes. Excess inoculum was removed and five mL of appropriate overlay medium (agar or liquid) was added. The agar overlay medium consisted of the following final concentration of ingredients: 2X MEM; 0.2% $NaHCO_3$; 100 I.U./mL P; 2.0 µg/mL G; 30 mM $MgCl_2$; 5.0% tryptose phosphate broth (Flow Laboratories, Mississauga, Ontario); 0.29 g/L glutamine; 0.6% purified agar (GIBCO, Burlington, Ontario), 2.0% heat inactivated FCS, and sterile distilled water. The agar was allowed to solidify at room temperature on a level surface. The cultures were then incubated at 37°C in an atmosphere of 5% $CO_2$ and 55% relative humidity. Controls, as described in Method I, were included in each assay. After 72 hours of incubation, one of each of the duplicate cultures was fixed and stained according to a procedure described by Holland et al. (9), while the remaining culture of each set was stained according to the University of Ottawa method described in Method I. The staining procedure of Holland et al. (9) was performed as follows. The agar overlay was removed from the culture dishes and the cell sheets were fixed and stained by adding 5 mL of fixative-dye solution (1% crystal violet in 20% ethanol) directly onto the agar surface. After two minutes, the excess dye was removed by washing in tap water and the cell sheets allowed to air dry. Plaques were counted on the same day.

## RESULTS

Plaques appeared as clear circular areas in a purple-stained cell sheet. Using method I, two sizes of plaques were observed. One type was medium sized, approximately 3 - 4 mm in diameter, while the other type was tiny (pin point). The average titer of the virus stock, using this method, and counting both sizes of plaques, was $1.2 \times 10^{8.5}$ plaque forming units

(pfu)/mL. Method II gave rise to pinpoint plaques only. Using this latter method, the average titer of the virus stock was $2.1 \times 10^{8.5}$ pfu/mL. Both staining methods were adequate and resulted in well stained cell sheets and plaques which were clearly visible.

Initially, no plaques were obtained using method I. Consequently, a number of media components were individually examined to determine whether any of them was responsible for cell toxicity or plaque inhibition. Experiments were performed in which varying concentrations of BUDR, DEAE-dextran and $MgCl_2$, respectively, were added to liquid overlay media of both inoculated and uninoculated cultures, or, were omitted completely. Neither BUDR nor $MgCl_2$ caused any apparent inhibition of cell growth or virus replication in the range of concentrations examined, including concentrations the same as, and greater than, those used in the agar overlay medium. DEAE-dextran, however, was found to be toxic to L 132 cells in liquid media at concentrations ranging from as little as 25 µg/mL (1/10th of that used in the actual plaque assay) to 1000 µg/mL, and to inhibit poliovirus cytopathic effect in concentrations >100 µg/mL.

Four different types of agar were evaluated for their effects on plaque production. There was no difference in the quality or quantity of plaques obtained when purified agar (Oxoid, Med-Ox Chemicals, Ottawa, Ontario), agarose (Marine Colloids Division, FMC Corp, Rockland, Maryland), or ionagar #2 were incorporated in the overlay medium. However, when a factory prepared purified agar suspension (GIBCO, Burlington, Ontario) was used, plaques were obtained which were marginally larger than those obtained with the other agar types examined.

During the course of the development of plaque assay systems for

poliovirus, several different types of cell culture Petri dishes were evaluated for their suitability in the assay. Linbro® multi-dish culture dishes did not support formation of cell monolayers. Few cells attached to the dish surface with the result that most remained suspended in the medium. Falcon®dishes did support healthy monolayers; however, the seeded cells tended to accumulate around the edges of the dish resulting in uneven growth of monolayers and cell sheets which were heavily populated around the edges but sparse in the center. In addition, the virus inoculum tended to accumulate at the edges. LUX Contur® culture dishes were found to be the most suitable. Homogeneous monolayers were formed across the whole dish surface and fluid inocula remained evenly in contact with the total cell sheet.

## DISCUSSION

Using Method I, two sizes of plaques (medium sized and tiny) were observed. Under normal conditions, plaques obtained from a virus preparation which contains only one virus serotype or strain should be equal in size. The fact that our virus stock was found to contain two virus serotypes (polio type 1 and 3), may account for our observation of two plaque sizes. Using Method II, only tiny plaques were observed. This observation could be accounted for if the media conditions of Method II were suitable for plaque production by only one of the two virus serotypes contained in the virus stock. A limited number of trials was conducted in which Method I was compared to Method II, using the same virus stock as source of inoculum and identical experimental conditions. When both medium and tiny sized plaques were counted in cultures prepared according to Method I, the number of plaques observed for a given dilution was approximately half of the number of tiny plaques observed for the same

dilution using Method II. The factors contributing to our observations of two plaque sizes and the descrepancies in plaque count are not clearly understood and further experiments using virus stock which is homogeneous for polio type 1, are required.

Both of the staining methods which were evaluated produced equally acceptable results. However, in the method as described by Holland et al. (9), fixation and staining are accomplished in one step. For this reason, we found this procedure to be more convenient and less time-consuming.

DEAE-dextran was found to be toxic to L 132 cells in liquid overlay at concentrations $\leq$ 2 µg/mL. DEAE-dextran is often added to agar overlay media to eliminate virus-inhibitory properties present in some agars. These inhibitors have been identified as polyanions, specifically, sulfated polysaccharides, and act by combining with virus particles, forming non-infectious virus-inhibitor complexes (10). DEAE-dextran, a polycation, acts by combining with the polyanion, thus neutralizing its inhibitory effect. Since, in liquid overlay media there are no polyanions to neutralize the effects of DEAE-dextran, the polycations are available to react with other charged substances, including host cells. In view of these findings, the observation in our experiments of gross cytotoxicity associated with the use of DEAE-dextran in liquid overlay, is not surprising.

In an evaluation of a number of commercially available culture dishes for their suitability for plaque assays, LUX Contur® plates were found to be superior. These dishes, unlike the others tested, are designed with curved edges to minimize the effects of meniscus tension at the fluid-plastic interface. Thus cells in liquid media do not tend to accumulate

e edges, but are distributed evenly over the whole surface of the

## SUMMARY

1. Two plaque assay systems (Methods I and II) have been developed for the quantitation of infectious poliovirus.

2. In order to evaluate the relative merits of the two methods, with respect to quality and quantity of plaque production, as well as efficiency of reagents, labor and time, more experimentation is indicated.

3. DEAE-dextran may be used in agar overlay media but should not be used in liquid overlay media.

4. LUX Contur® culture dishes are highly suitable for virus plaque assays.

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